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RNA Loading of Leukemic Antigens into Cord Blood–Derived Dendritic Cells for Immunotherapy

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ABSTRACT

The manipulation of dendritic cells (DCs) ex vivo to present tumor-associated antigens for the activation and expansion of tumor-specific cytotoxic T lymphocytes (CTLs) attempts to exploit these cells' pivotal role in immunity. However, significant improvements are needed if this approach is to have wider clinical application. We optimized a gene delivery protocol via electroporation for cord blood (CB) CD34$^+$ DCs using in vitro–transcribed (IVT) mRNA. We achieved > 90% transfection of DCs with IVT-enhanced green fluorescent protein mRNA with > 90% viability. Electroporation of IVT-mRNA up-regulated DC costimulatory molecules. DC processing and presentation of mRNA-encoded proteins, as major histocompatibility complex/peptide complexes, was established by CTL assays using transfected DCs as targets. Along with this, we also generated specific antileukemic CTLs using DCs electroporated with total RNA from the Nalm-6 leukemic cell line and an acute lymphocytic leukemia xenograft. This significant improvement in DC transfection represents an important step forward in the development of immunotherapy protocols for the treatment of malignancy.

KEY WORDS

Dendritic cells • Electroporation • Immunotherapy • Cytotoxic T lymphocyte

INTRODUCTION

Interest in active and adoptive cellular immunotherapy to treat cancer has increased with recognition of the pivotal role of dendritic cells (DCs) in the initiation of adaptive immune responses [1,2]. Preparation of DCs to present specific tumor-associated antigens (TAAs) for phase I clinical trials have proven the safety of this approach, and some limited but encouraging successes have been reported [3-8]. These trials have reinforced the potential for the immune system to target and eradicate cancer using cytotoxic T lymphocyte (CTL) effectors generated by autologous DC stimulation either in vivo or in vitro.

There are strong clinical incentives for refining DC generation from CD34$^+$ hematopoietic progenitor cells, particularly from CBs, which are increasingly being used as a stem cell source in transplantation given their ready availability and the fact that outcomes for CB transplant recipients compare favorably with those for matched sibling bone marrow transplant recipients. The capacity to engineer DCs from CB is likely to play an increasingly important role in strategies to promote a graft-versus-leukemia (GVL) effect in a manner analogous to the use of child/adult donor leukocyte infusions after transplantation [9]. Using DCs to generate antileukemic CTLs to treat relapsed acute lymphoblastic leukemia (ALL) after

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hematopoietic stem cell transplantation (HSCT) is particularly relevant, because most CB transplantations to treat this disease are performed in children. With the shift toward CB as a stem cell source for HSCT comes the need to ensure that adoptive immunotherapy post-HSCT is feasible with CB-derived cells. We propose that anti-ALL CTL generated in vitro, administered after they have engrafted, is a potentially effective adoptive immunotherapy to prevent leukemic relapse.

RNA, as a source of antigen, is an efficient way to introduce antigens into DCs and is useful when the target is relatively nonimmunogenic, as in ALL. Electroporation introduces RNA into Monocyte-derived DCs (MoDCs) for the generation of antigen-specific CTLs [10-15]. Blood DCs also process and present antigen after electroporation with RNA. The electroporation settings used routinely kill a significant proportion of DCs, and in those that survive, variable transfection efficiency may dramatically affect their ability to process and present antigen. We therefore sought to systematically optimize the RNA loading of CB $CD34^+$-derived DCs ($CD34^+$DCs) to generate CTLs.

Mature CB-derived $CD34^+$DCs were generated and reproducibly achieved $> 90\%$ transfection efficiency with in vitro–transcribed (IVT)-enhanced green fluorescent protein (EGFP) mRNA while retaining $> 90\%$ viability. We showed that electroporation of mRNA up-regulated $CD34^+$DC costimulatory molecules and that the translated protein was processed and presented in the context of major histocompatibility complex (MHC) molecules. Using total RNA from the leukemic cell line Nalm-6 and an ALL xenograft, we were able to generate specific antileukemic CTLs. Achievement of this high-level reproducible transfection efficiency and successful CTL generation represents an important step forward in the development of clinical immunotherapy protocols to treat ALL and other malignancies.

MATERIALS AND METHODS

Cord Blood

The research ethics committees of the Mater Misericordiae Hospitals and University of New South Wales approved the use of human CB for research purposes. CB samples were obtained after normal full-term delivery of babies with the mothers' informed consent. CB was processed within 24 hours of collection.

CB $CD34^+$ Stem Cell Isolation

$CD34^+$ cells were positively selected from the mononuclear cell (MNC) fraction with MACS beads (Miltenyi Biotech), as described previously [16]. $CD34^+$ hematopoietic progenitor cells and $CD34^-$ MNC fractions were cryopreserved and stored in liquid nitrogen.

Generation of $CD34^+$DCs

Cryopreserved purified $CD34^+$ cells were thawed and resuspended at 2×10^4 $CD34^+$ cells/mL in phenol red-free, X-VIVO 15 serum-free medium (Cambrex) containing glutamine, penicillin, and streptomycin (Invitrogen) as base medium supplemented with 35 ng/mL of granulocyte macrophage colony-stimulating factor (Novartis), 50 ng/mL of FLT3-L (Amgen), 50 ng/mL of SCF (Amgen), 1 ng/mL of tumor necrosis factor-α (R&D Systems), and 0.5 ng/mL of transforming growth factor-β (R&D Systems). Half of the cytokine medium was changed on days 5, 7, 9, and 11. Cells were harvested for electroporation studies on days 7 and 9.

Flow Cytometry

Phenotypic analysis of DCs after expansion in cytokine medium was determined by flow cytometry. A total of 10,000 events were routinely collected on a FACS Calibur flow cytometer (BD Biosciences, San Jose, CA), and the results were analyzed with CELLQuest (BD Biosciences) or FCS Express (De Novo Software, Thornhill, Ontario, Canada). $CD34^+$ stem cell purity was determined with CD3 (fluorescein isothiocyanate [FITC]), CD14 (Phycoerytherin [PE]), CD34 (PerCP-Cy5.5), and CD45 (Allophycocyanin [APC]) antibodies (BD Biosciences). Antibodies for DC phenotype analysis included CD1a, CD40, CD80, CD83 (all PE), CD11c (APC), HLA-DR (PerCP-Cy5.5), lineage markers (CD3, 14, 19, 20, and 56, all FITC), and isotype controls (mouse-IgG1 FITC, PE, PerCP-Cy5.5, and APC) (BD Biosciences), CD86 (PE), CD15 and CD54 (FITC) (BD Biosciences or Immunotech), and BDCA-2 (CD123, PE) (Miltenyi). Cell viability was determined with 7-AAD (Immunotech). HLA-A2 status of CB was determined using HB-B2 hybridoma supernatant and sheep anti-mouse immunoglobulin FITC (Chemicon).

For comparative experiments, when up-regulation of DC activation markers was investigated, the increase in mean fluorescence index (MFI) was measured as a surrogate marker of antigen expression. The increase in MFI was calculated by

$$\text{Fold MFI} = (\text{sample MFI} - \text{isotype control MFI})/\text{isotype control MFI}.$$

Plasmids

The vectors pGEM®4Z/FMP/64A encoding influenza matrix protein (FMP) and pGEM®4Z/EGFP/64A encoding EGFP were provided by Professor Eli Gilboa, Duke University Medical Center. Vectors

were linearized after overnight incubation with Sure-cut buffer, water, and Spe I enzyme (Roche, Basel, Switzerland) at 37°C, followed by chloroform extraction. Linearized DNA was stored in Tris-EDTA buffer (10 mM Tris Cl and 1 mM EDTA, pH 8) at −70°C and used as required for IVT. The full-length human survivin cDNA (kindly provided by Professor Dario Altieri, University of Massachusetts Medical School) was subcloned into the *Hind*III/*Bam*HI site of the pGEM-4Z vector (Promega, Madison, WI). After large-scale amplification and purification, the plasmid was linearized with *Bam*HI and used as a template for IVT.

In Vitro Transcription of mRNA

A MEGAscript High-Yield Transcription Kit (Ambion, Austin, TX) was used for the IVT of survivin mRNA. During the reaction, the guanosine triphosphate concentration was adjusted to 1.5 mM, and the cap analog $m^7G(5')$ ppp (5′)G (Ambion) was included at 6 mM, with adenosine triphosphate, cytidine triphosphate, and uridine triphosphate concentrations at 7.5 mM. Template DNA was digested with RNase-free DNase I, and RNA was recovered by LiCl/ethanol precipitation. The RNA pellet was dried in air, resuspended in sterile water, and incubated for 30 minutes at 30°C in 20 mM of Tris.Cl (pH 7.0), 50 mM of KCl, 0.7 mM of $MnCl_2$, 0.2 mM of EDTA, 100 mg/mL of acetylated bovine serum albumin, 10% glycerol, 1 mM of adenosine triphosphate, and 5,000 U/mL of yeast poly(A) polymerase (USB, Cleveland, OH). The capped, polyadenylated RNA was recovered as described earlier, quantified, and stored at −80°C.

Transcription of EGFP- and FMP-linearized DNA was performed using the mMessage mMachine T7 kit (Ambion) according to the manufacturer's instructions to generate IVT-mRNA. mRNA purification was performed by elution using the MEGAclear kit (Ambion). RNA concentration was calculated by spectrophotometry, and the quality and size were checked by gel electrophoresis. IVT-mRNA was stored in aliquots at −70°C.

Total RNA Extraction

Total RNA from the Nalm-6 cell line and an ALL xenograft (grown in NOD-SCID mice exactly as described previously [17]) was extracted using the Qiagen RNeasy Midi kit (Qiagen, Hilden, Germany), according to the manufacturer's instructions. A total of 10^8 cells were lysed and homogenized. Ethanol was added to promote binding of RNA to the RNeasy silica gel membrane, and the sample was washed. The quality and quantity of RNA were determined by spectrophotometry and gel electrophoresis. Total RNA was stored at −70°C.

Transfection Protocols

$CD34^+$ hematopoietic progenitor cells (HPC) cultures were harvested on days 7 or 9 (as specified in the text) and centrifuged to remove the cytokine medium before being resuspended in electroporation medium and subjected to electroporation in 200-μL cuvettes in a Gene Pulser II (BioRad, Hercules, CA). Optimization experiments investigated the following variables: range of voltage (50–350 V), range of capacitance (75-150 μF), electroporation medium (X-VIVO 15, RPMI medium 1640 and Opti-MEM [Invitrogen, San Diego, CA), incubation of cells before (10 minutes) and after electroporation (0–30 minutes) at varying temperatures (37°C, ice, room temperature), mRNA concentration (2–16 μg), and cell concentration (1–20 × 10^6/mL). EGFP expression and cell viability were determined by flow cytometry performed 24 hours postelectroporation after cells were stained with 7-AAD for viability and HLA-DR and CD11c for $CD34^+$DC. Transfection efficiency was evaluated in total cell and DC subpopulations.

CTL Generation

$HLA\text{-}A2^+$ DCs were electroporated on day 9 of culture with 20 μg of Nalm-6 total RNA or 20 μg of total RNA ALL-19 xenograft or 8 μg of survivin IVT-RNA per 1 × 10^6 cells. Then, 12 hours after transfection, autologous $CD34^-$ MNCs were primed with transfected DCs at 2 × 10^6 MNCs: 2 × 10^5 DCs/well in X-Vivo 15 medium supplemented with 25 U of interleukin (IL)-2/mL (Roche) and 25 ng of IL-7/mL (PeproTech, Rocky Hill, NJ). Fresh medium and 25 U/mL of IL-2 and 25 ng/mL of IL-7 were added every 3 days. The remaining transfected DCs were cryopreserved for restimulation. At restimulation, autologous transfected DCs were thawed and used as stimulators at an Effector: Target (E:T) ratio of 10:1. A chromium release assay was performed every 7 days.

^{51}Chromium Release Assay

A standard 4-hour ^{51}Cr assay was performed to assess the specificity of CTLs. Target cells were washed in RPMI-1640 with 10% fetal calf serum and labeled with 100 μC ^{51}Cr (Amersham BioSciences, Piscataway, NJ) for 1 hour at 37°C. Effector cells were prepared in RPMI-1640 with 10% fetal calf serum. The final volume of effectors and targets was 100 μL in 96-well V-well plates with appropriate controls for spontaneous and maximum release of ^{51}Cr. K562 cells were included as cold targets to remove natural killer cell killing. After 4 hours of incubation at 37°C, the plates were centrifuged, and 50 μL of supernatant was harvested and added to the Optimix (OptiPhase Supermix, Wallac, Finland) scintillation fluid. ^{51}Cr release was read on a Wallac MicroBeta TriLux liquid scintillation and luminescence counter

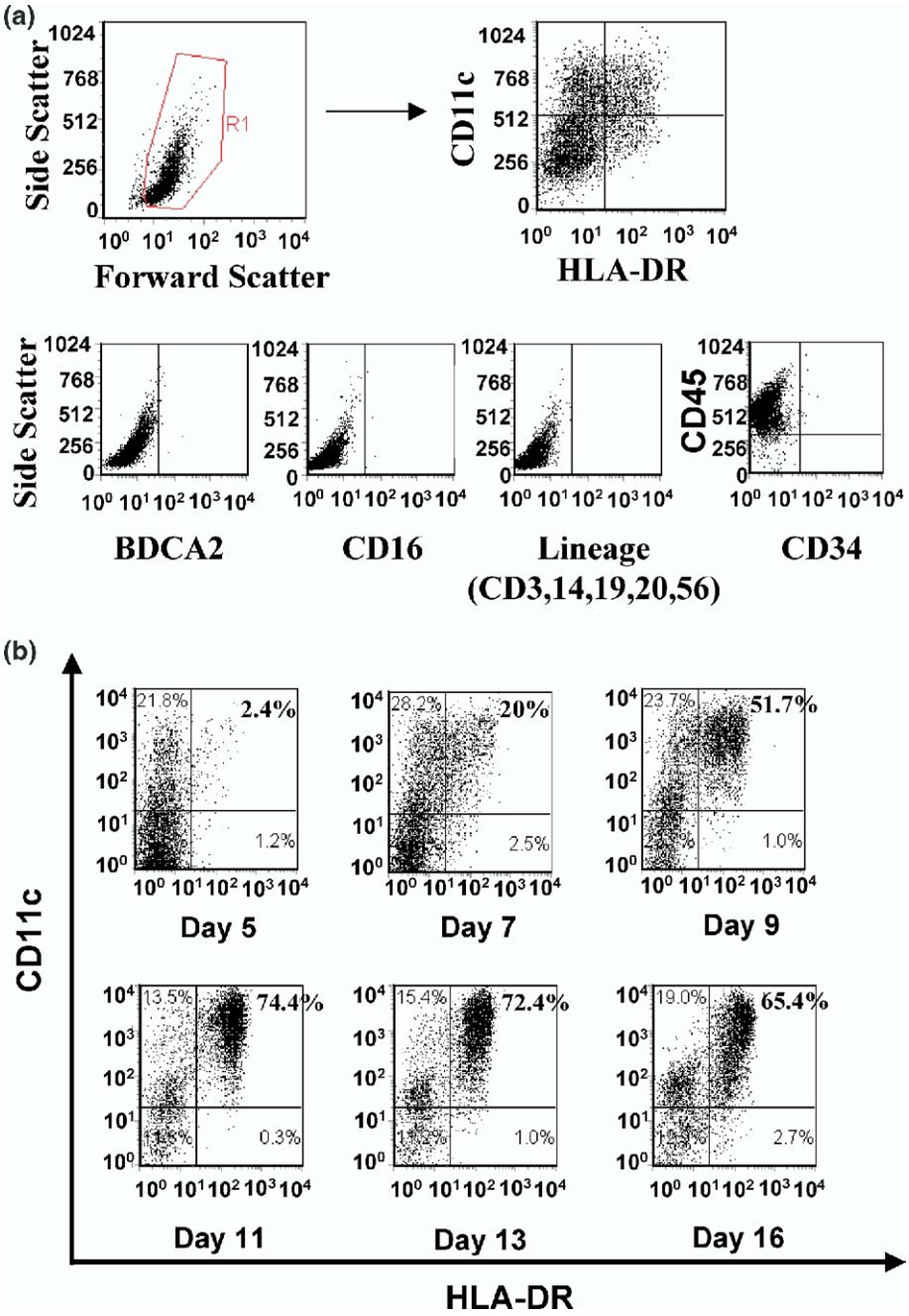

Figure 1. Characterization of CB-derived DCs. (A) Subset analysis of cell cultures on day 9 followed initial gating on the live cell gate (R1), then staining for HLA-DR and CD11c. The cells could then be subdivided to identify the DC subset in the upper right quadrant (DR^+11c^+). Cells in the upper left quadrant (DR^-11c^+) were designated precursor or immature DCs, and those in the lower left quadrant (DR^-11c^-) were designated non-DCs. The next 4 panels show that the cultures were BDCA2, CD16, lineage (CD3, CD14, CD19, CD20, and CD56), and CD34 negative. (B) The DR/11c phenotype of the culture was analyzed over time. (C) Expression of costimulatory and activation markers (CD40, CD54, CD80, CD83, and CD86) on all 3 populations over a time course from day 7 to day 10.

(PerkinElmer, Boston, MA). Specific lysis was calculated according to the following standard formula:

$$\%\ \text{specific lysis} = (\text{counts in test well} - \text{counts in spontaneous wells})/(\text{counts in maximum lysis well} - \text{counts in spontaneous wells}) \times 100$$

Statistical Analysis

Systat 10.2 software (Hearne Scientific, Melbourne, Australia) was used for experimental design and analysis. All experiments were repeated at least 3 times, and results are expressed as mean ± standard error of the mean. Results were significant at $P \leq .05$. Analysis of variance (ANOVA) and post hoc Bonfer-

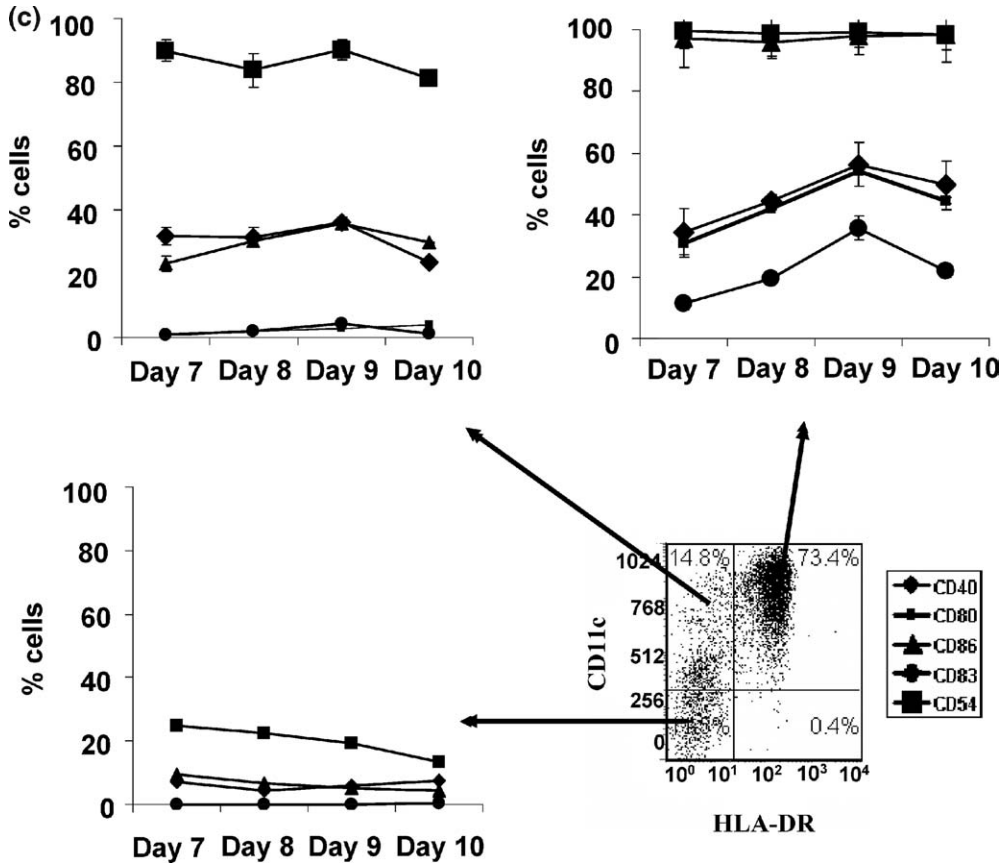

Figure 1. (Continued)

roni tests were used to determine the optimal cytokine combination to generate DCs and to identify which electroporation variables had a significant effect on EGFP expression and cell survival.

RESULTS

Generation of CD34$^+$DCs

CD34 expression was lost by day 7 of culture, and the cultured cells lacked lineage marker expression (CD3, CD14, CD19, CD20, and CD56) as well as the CD16, CD123, and BDCA2 antigens (Figure 1A). Most of these CD34$^+$DC preparations have the phenotypic characteristics of DCs (HLA-DR$^+$ CD11c$^+$Lin$^-$). Differential expression of CD11c and HLA-DR by the CD34$^+$DCs enabled discrimination of 3 distinct populations, designated as precursor or immature DCs (CD11c^{hi}HLA-DR^{low}), mature DCs (CD11c^{hi}HLA-DR^{hi}), or non-DCs (CD11c$^-$HLA-DR$^-$) (Figure 1B). Total cell counts (50 ± 5 total cells per CD34$^+$-initiating cell at day 7 [n = 10] and 62 ± 6 total cells per CD34$^+$-initiating cell at day 9, [n = 22]) indicated a major increase in total cell number by day 7 but a slower rate of expansion thereafter. A single CD34$^+$-initiating cell gave rise to 19 ± 3.9 CD34$^+$DCs (n = 8) by day 7 of culture and 40 ± 1.8 CD34$^+$DCs (n = 12) by day 9 of culture. Subset analysis of activation and costimulatory molecules known to effect DC function (CD40, CD54, CD80, CD83, and CD86) revealed that non-DC and immature/precursor populations expressed lower levels than the mature DC subset. Evaluation of their expression patterns on days 7–10 of culture showed that peak expression occurred on day 9 in the mature DC subset (Figure 1C). These findings provided a rational basis for performing most electroporation experiments on CD34$^+$DCs on day 9.

Preliminary Transfections

Electroporation parameters commonly used for MoDCs (300–350 V and 100–150 μF) [10,11,13,14,18] were used to determine whether these conditions could be translated to CD34$^+$DCs on days 7 and 9 of culture. Using these conditions, we achieved only 30% ± 5% (n = 5) EGFP transfection efficiency of the DC subset with poor viability (20% ± 1.5%; n = 5). Light microscopy revealed extensive cell damage as soon as 6 hours after electroporation; therefore, we examined the key variables in the electroporation protocol in an effort to improve both transfection efficiency and cell viability.

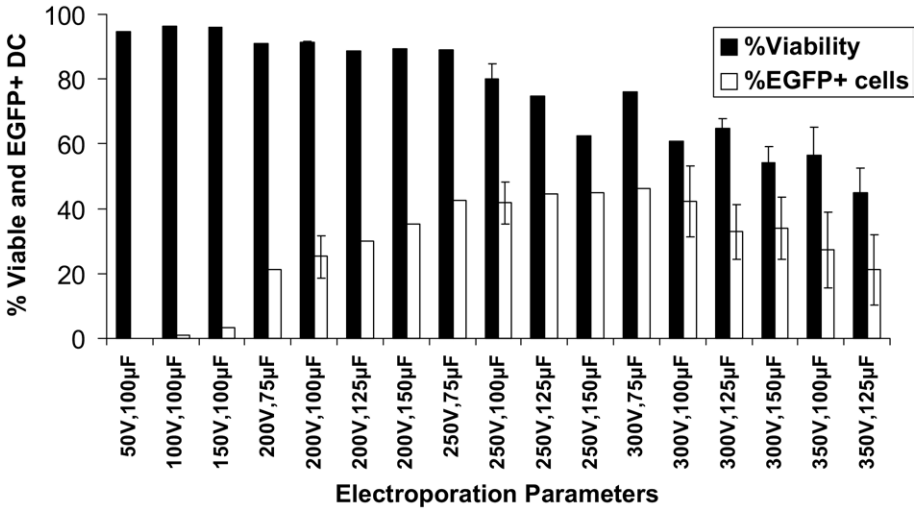

Figure 2. Transfection efficiency and viability of DCs over a range of voltage and capacitance settings. $CD34^+$ stem cell cultures were harvested on day 9 and transfected in X-Vivo 15 with 4 μg of IVT-EGFP mRNA. The voltage ranged from 50 to 350 V, and capacitance ranged from 75 to 150 μF. Flow cytometric analysis was performed 24 hours postelectroporation using HLA-DR and CD11c to identify the DC population. The graph shows the percentage of viable DCs (black bar) and the percentage of viable DCs expressing EGFP (white bar).

Electroporation Voltage and Capacitance

Day 9 $CD34^+$DC cultures were electroporated with EGFP IVT-mRNA in X-VIVO 15 medium over a range of voltages (50–350 V) and capacitances (75–150 μF) and incubated on ice for 1 minute before being returned to cytokine-containing medium. Increasing the electroporation settings resulted in greater cell death with low to moderate transfection efficiency, whereas reducing these settings produced greater cell viability, but also lower transfection efficiency (Figure 2). Light microscopy examination of $CD34^+$DCs revealed extensive cellular damage and debris at higher settings (350 V; 125 μF) than at lower settings (250 V; 100 μF). These experiments also showed an electromagnetic membrane permeation threshold of 100 V for $CD34^+$DCs, with virtually no EGFP expression below this voltage. Above this threshold, a range of settings (250–300 V with 75–100 μF) resulted in greater EGFP expression with moderate cell survival. The next step was to improve DC transfection efficiency and viability by investigating the effect of other parameters likely to affect cell integrity.

Electroporation Media, Incubation, and Temperature

Cell survival and transfection efficiency were compared for cells electroporated in X-VIVO 15, RPMI-1640, and Opti-MEM media at 300 V and 125 μF. A preliminary matrix experiment demonstrated that cells electroporated in RPMI-1640 had higher EGFP transfection efficiency. Viability decreased with prolonged incubation on ice after electroporation regardless of the media used (Figure 3A). The time constants (ie, the time [in seconds] for the initial electromagnetic pulse to decay to 63% of its initial field strength) recorded for these electroporation events were highly consistent for each medium (RPMI-1640: 1.96 ± 0.06 seconds; X-VIVO 15, 2.35 ± 0.06 seconds; Opti-MEM, 2.28 ± 0.03 seconds; n = 12). ANOVA confirmed that media had a significant effect on the electroporation time constant ($P < .001$), suggesting that a lower time constant had a positive effect on cell survival. Post hoc Bonferroni tests revealed that the RPMI-1640 medium permitted the greatest cell survival ($P = .02$), although recovery was still less than desirable. In subsequent experiments, RPMI-1640 was used as the medium for electroporation. Lower voltage and capacitance settings (250 V and 100 μF) were also applied to maximize cell viability.

We investigated how maintaining cells at a specific temperature before or after electroporation affected cell survival and transfection efficiency. Prolonged incubation at low temperature after electroporation resulted in a drop in viability compared with incubation at room temperature (Figure 3B). The inclusion of an additional 10-minute incubation at 37°C before electroporation, followed by 5 minutes at room temperature after electroporation, further improved viability (> 90%; $P = .026$ for preincubation at 37°C and $P = .025$ for postelectroporation at room temperature) (Figure 3C).

mRNA and Cell Concentration and Time Course of mRNA Expression

Ideally, all cells should be equally exposed to the electrical field to achieve maximum mRNA uptake.

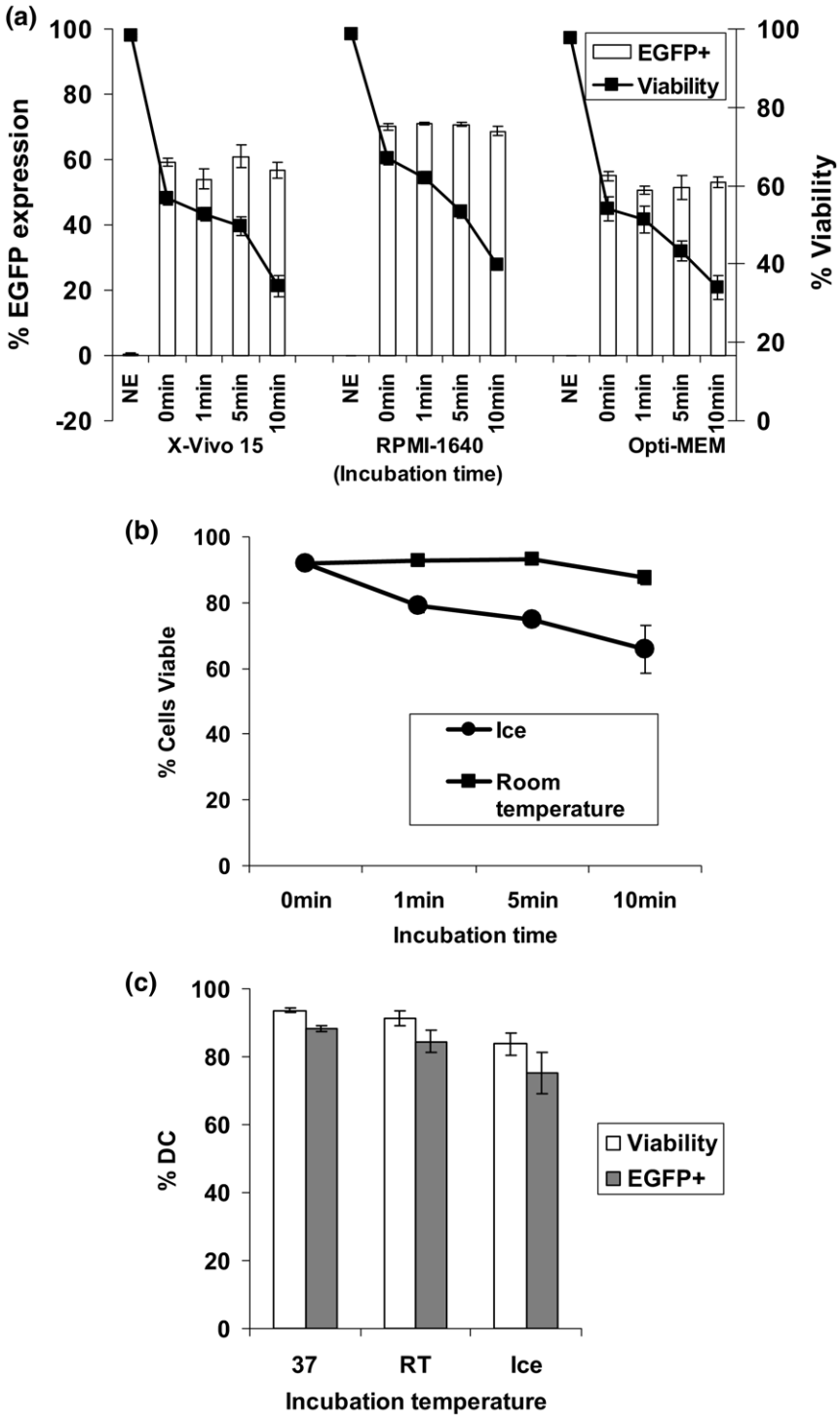

Figure 3. The effect of electroporation medium, incubation time, and temperature on transfection efficiency and cell survival. (A) $CD34^+$ stem cell cultures were harvested and electroporated (at 300 V and 125 μF) on day 9 of culture in RPMI-1640, Opti-MEM, or X-VIVO 15 with 4 μg of IVT-EGFP mRNA. Electroporated cells were then incubated in the electroporation medium (either RPMI-1640, Opti-MEM, or X-VIVO 15) for up to 10 minutes on ice before being returned to the cytokine culturing medium. Viability (7-AAD exclusion) and EGFP expression were assessed by flow cytometry 24 hours later. (B) Cells were electroporated under the same conditions just described (in RPMI-1640), except the cells were incubated postelectroporation for up to 10 minutes at room temperature or on ice, and viability was assessed by 7-AAD exclusion. (C) Cells were incubated either at 37°C, on ice or at room temperature for 10 minutes before electroporation. After electroporation, cells were incubated for a further 5 minutes at room temperature before being returned to the cytokine culturing medium.

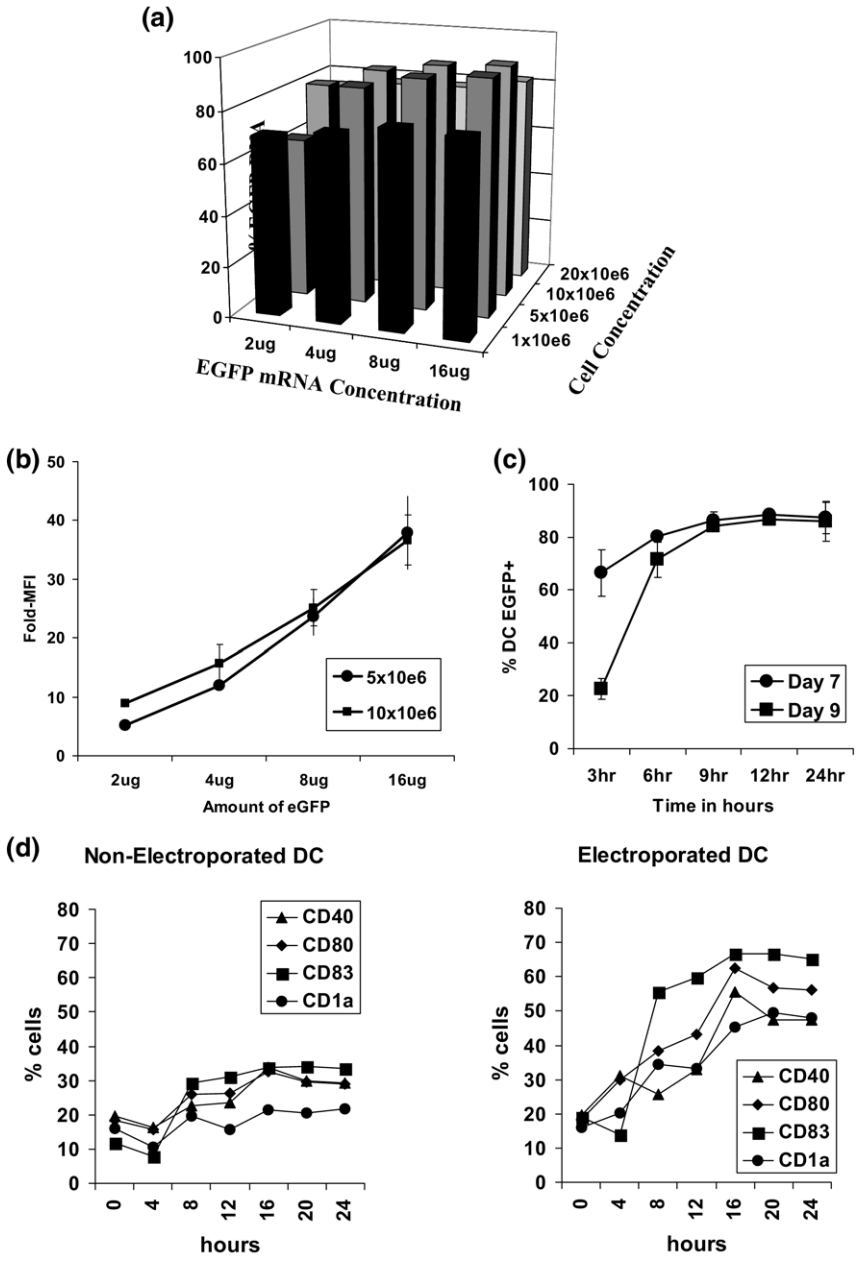

Figure 4. The effect of varying RNA and cell concentration on transfection efficiency and time analysis of EGFP expression, and effect of RNA on DC costimualtory capacity. $CD34^+$ cells were harvested on day 9 and electroporated at 250 V and 100 μF in RPMI-1640. DC viability and EGFP expression were analyzed 24 hours postelectroporation. (A) Cells at concentrations of 1–20 × 10^6/mL were electroporated with 2–16 μg of IVT-EGFP. (B) Fold-MFI analysis of EGFP expression of DC electroporated at 5 or 10 × 10^6/mL, with mRNA amounts ranging from 2 to 16 μg. (C) Using the same settings as before, 5 × 10^6/mL of cells were electroporated on days 7 and 9, and DC EGFP expression was analyzed over a time course. (D) Effect of electroporation and RNA on DC costimulatory capacity. $CD34^+$ cell cultures were harvested on day 9 and electroporated with patient ALL total RNA. Over 24 hours, CD1a, CD40, CD80, and CD86 were analyzed.

We performed a matrix experiment comparing the effect of mRNA concentrations and cell numbers on transfection efficiency using optimal settings and conditions. Cell concentrations of 5 × 10^6/mL and 10 × 10^6/mL had the highest transfection efficiencies (Figure 4A), but all cell preparations had viability > 90% (data not shown). Increasing mRNA concentration had a significant effect on the proportion of $EGFP^+$ cells within the whole cell population (ANOVA; $P < .001$). The DC population appeared to be selectively targeted and reached a maximum transfection efficiency plateau at 4–8 μg (data not shown); however, MFI analysis of these DCs at 5 × 10^6/mL and 10 × 10^6/mL showed that increasing mRNA concentration correlated with increased EGFP protein production (ANOVA; $P = .001$) (Figure 4B).

We examined EGFP expression over an extended time course, because this is likely to have a major

effect on downstream in vitro and in vivo CTL generation. Using the same parameters (5×10^6 cells/mL and 4 μg mRNA), we performed a 24-hour EGFP readout for cells electroporated on days 7 and 9. EGFP expression was readily detected 3 hours after electroporation and peaked at around 12 hours for both day 7 and 9 $CD34^+$DCs (Figure 4C).

Because our clinical goal was to generate antileukemic CTLs from leukemic RNA-loaded $CD34^+$DC, we monitored the up-regulation of CD1a, CD40, CD80, and CD83 expression by $CD34^+$DCs after electroporation with total RNA derived from a patient ALL xenograft. $CD34^+$DC expression of CD1a, CD40, CD80, and CD83 was up-regulated and reached a plateau at or after 16–20 hours postelectroporation compared with nonelectroporated DCs (Figure 4D). The expression of CD86 remained high (in > 90% of $CD34^+$DCs) regardless of electroporation; thus, we determined that incubation for 12 hours would maximize antigen processing and presentation by electroporated $CD34^+$DCs before co-culturing with T cells for CTL induction.

Optimized Transfection Protocol

We compared transfection efficiencies obtained after optimizing the procedure with those obtained with the starting conditions derived from the literature (Figure 5). We found that up to 96% (92.32% ± 0.73%; n = 6) transfection efficiency, accompanied by high viability (> 93%), was achievable in $CD34^+$DCs using our optimized protocol. Thus, when using 5×10^6 total $CD34^+$DCs for electroporation, we would expect to obtain 2.5×10^6 live $EGFP^+$ $CD34^+$DCs, which is sufficient to stimulate 25×10^6 MNCs for CTL induction and produce 100×10^6-expanded CTLs by 2 weeks. These electroporation conditions have also been tested on BDCA-1^+ blood DCs with similar results (data not shown).

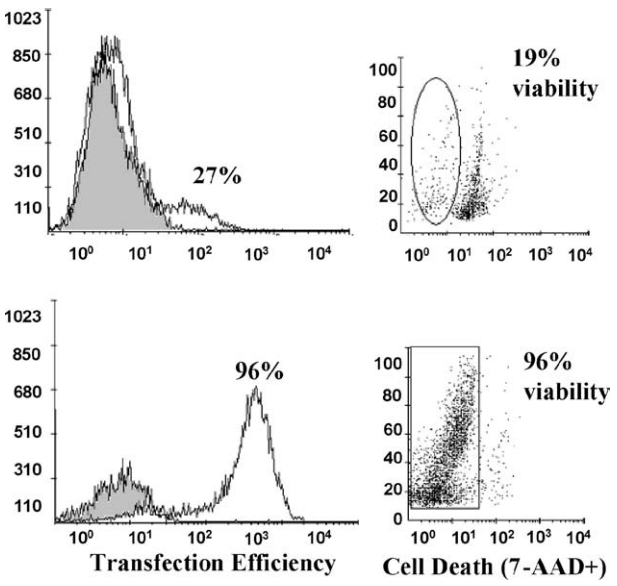

Figure 5. Comparison of nonoptimized and optimized electroporation conditions. Comparison of transfection efficiency and cell viability (24 hours postelectroporation) of $CD34^+$ stem cell–derived DCs before and after optimization. (A) Electroporation using nonoptimized conditions (300 V, 125 μF) followed by 1 minute of incubation on ice. (B) Electroporation with the following parameters: preincubation of 5×10^6 cells at 37°C for 10 minutes, 250 V, 100 μF, electroporation in RPMI-1640 with 4 μg of IVT-EGFP, incubation at room temperature for 5 minutes postelectroporation.

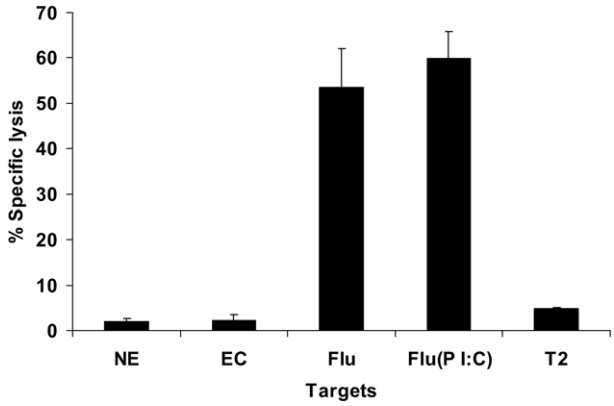

Figure 6. Presentation of FMP peptides. $CD34^+$ HSC culture was harvested on day 9 and transfected with IVT-FMP (Flu) mRNA under the optimized conditions. Then, 24 hours after electroporation, cells were labeled with ^{51}Cr for use as targets in CTL assays. Effectors were FMP-T cell clones (specific for epitope GILVFTFTL), and the E:T ratio was 10:1. Targets were nonelectroporated (NE) controls, electroporated cells without FMP electroporated control (EC), T2 controls, FMP-transfected cells, and FMP-transfected DCs with the addition of Poly I:C for 24 hours. ^{51}Cr release was measured.

Presentation of Peptides on $CD34^+$DCs in the Context of MHC Molecules

To confirm that transfected mRNA was translated and the protein was processed and presented as peptides in the context of MHC molecules by the $CD34^+$DC, we examined the susceptibility of day 9 FMP-mRNA transfected $CD34^+$DCs as CTL targets for HLA-A201–matched FMP-specific T-cell clones (E:T ratio of 10:1). FMP-mRNA was translated and presented on the MHC, as evidenced by specific CTL lysis (54% ± 8%; n = 3) of day 9 FMP-RNA–loaded $CD34^+$DCs (Figure 6). The addition of polyinosine-polycytidylic acid (poly I:C) as a DC maturation agent induced only a marginal further increase in specific lysis (58% ± 6%; n = 3), consistent with our phenotypic data suggesting that $CD34^+$DCs already express activation and costimulatory molecules (Figure 1C).

Generation of Antileukemic CTLs

Day 9 $CD34^+$DCs from 3 HLA-$A2^+$ CB donors were electroporated with Nalm-6 total RNA to generate bulk CTL cultures. After 1 antigenic restimula-

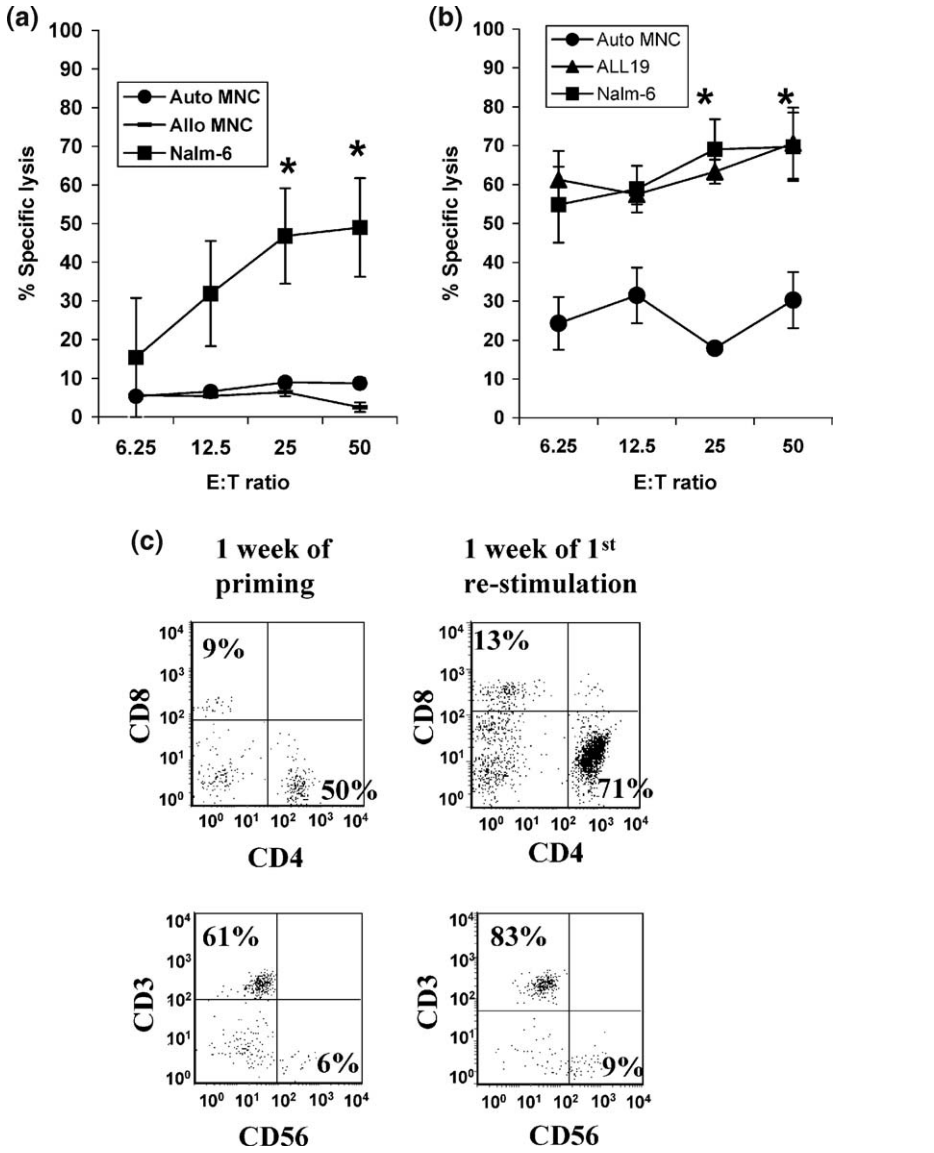

Figure 7. CTLs generated from total RNA electroporated DCs are able to specifically lyse leukemia targets. (A) HLA-A2$^+$ DCs from 3 donors were harvested on day 9 of culture and electroporated with Nalm-6 total RNA. Autologous MNCs were primed with the electroporated DCs, and ^{51}Cr release assays were conducted after the first restimulation with thawed autologous DC electroporated with Nalm-6 total RNA. Targets used were Nalm-6 and autologous and allogeneic MNCs (50:1 CTL to autologous, $P = .03$; to allogeneic, $P = .022$; 25:1 CTL to autologous, $P = .029$; to allogeneic, $P = .021$). (B) A2$^+$ DCs from 2 donors were used to generate bulk CTLs with patient ALL total RNA. The targets used were autologous MNCs, Nalm-6, and autologous ALL. (C) Phenotype of bulk cultures (CD3, CD4, CD8, and CD56) 1 week after priming and after the first restimulation.

tion, the CTLs specifically lysed Nalm-6 targets at 48% ± 11% at an E:T ratio of 50:1, compared with 8.5% ± 1.3% for autologous MNCs ($P = .03$) and 2.5% ± 1.2% for allogeneic HLA-A2$^+$ MNCs ($P = .022$) (Figure 7A).

CTLs generated using this system also recognized primary leukemia cells. Total RNA derived from an HLA-A2$^+$ patient ALL xenograft that had been expanded and extracted from NOD-SCID mice [23] were used to load HLA-A2 CD34$^+$DCs. After a single antigenic restimulation, the anti-ALL xenograft CTLs lysed 63% ± 6% of the ALL xenograft cells, compared with 22% ± 7% of autologous MNCs ($P < .001$) (Figure 7B). The bulk CTL culture consisted of CD4 (68% ± 1.8%) and CD8 (12.6% ± 1.4%) T cells, which increased in number after a single in vitro restimulation, with the population of natural killer cells remaining at < 10% (Figure 7C). Interestingly, these CTLs also retained their capacity to lyse the HLA-A2$^+$–matched Nalm-6 cell line ($P < .001$). We also tested whether these anti-ALL xenograft CTLs were able to target generic proteins such as survivin (an inhibitor of apoptosis expressed in both leukemia and solid tumors [19]) by using autologous

$CD34^+$DCs electroporated with IVT-survivin mRNA as targets in the ^{51}Cr CTL assay. Here 16% ± 5.4% of survivin mRNA electroporated $CD34^+$DCs were lysed, compared with only 5%-7% of nonelectroporated DCs (data not shown). These observations suggest that using our optimized electroporation protocol, RNA-loaded $CD34^+$DCs are capable of generating antileukemic CTLs that specifically recognize leukemic cells and generic tumor-associated antigens.

DISCUSSION

Strategies that use DC-mediated induction of antigen-specific effector CTLs to promote a GVL effect posttransplantation may improve outcomes for patients undergoing transplantation for acute leukemia. Current strategies to treat leukemic relapse have used donor leukocyte infusions and second transplantation with limited success [20,21]. For DC-based immunotherapy to be effective in the posttransplantation setting, there must be reliable protocols for generating functional DCs from the transplant product and a reproducible and safe method for introducing TAAs into DCs. The increased use of CB as a transplant stem cell source has prompted us to develop and optimize protocols for the production of immunotherapy tools—namely DC and antigen-loading procedures—from CB $CD34^+$ cells. As a result, we have generated CB-derived $CD34^+$DCs that, when electroporated with leukemic RNA, stimulate the generation of antileukemic CTLs.

Successful electroporation of mRNA has been reported in monocyte-derived DCs (30%–60%) [10,14,15,22], bone marrow $CD34^+$-derived DCs (72%) [15, 22], Langerhans cells (53%) [15, 22], and peripheral blood $CD34^+$-derived DCs (89%) [23]. Successful electroporation of CB-derived DCs (70%–90%) also has been reported [24,25], but these studies used serum to generate the DCs. A number of these reports do not discuss cell viability after electroporation, and in those that do, the range is between 40% and 90% [15,22,23]. Our findings represent a significant step forward, because our $CD34^+$DC culture system is serum-free, and the optimized electroporation protocol results in consistently high transfection efficiency and viability.

Electroporation carries an inherent risk of irreversibly damaging the cell membrane and possibly killing the cell. A balance between the extent of pore formation, transfection efficiency, and the cell's ability to recover after transfection must be established. Pore formation depends directly on the strength of the applied voltage and capacitance, because it is responsible for the level and magnitude of cell membrane disruption that allows the entry of macromolecules into cells [26,27]. Increasing these settings correlated with higher transfection efficiency and lower cell viability. Our optimal settings (250 V and 100 μF) are lower than the conditions used for MoDCs. A possible explanation for this is that cell size determines the minimal electrical field strength required to permeate cell membranes, according to the formula $E = Vc/1.5\times A$, where E is the electrical field strength, Vc is the critical breakdown voltage, and A is the cell radius [26,28]. The media supporting the cells during electroporation affects the conductivity of the electromagnetic pulse, with its impact reflected in the time constant. A higher time constant implies that the applied pulse decays at a slower rate, exposing cells to the applied pulse for longer periods and possibly resulting in greater damage. The medium with the lowest time constant (RPMI-1640) produced the highest $CD34^+$DC survival. Incubating electroporated cells on ice caused unacceptably high cell death, probably by reducing cell metabolism and hence impairing cell repair. Thus, warming cells before electroporation at 37°C and incubating them at room temperature after electroporation can further improve transfection efficiency and viability.

Cell concentrations used for electroporation vary greatly ($1–50 \times 10^6$/mL) [18,24,25,29]. We showed that $CD34^+$DCs can be electroporated effectively at $1–20 \times 10^6$/mL, and that electroporation at higher cell concentrations in a clinical setting will be more efficient and more compatible with clinical Good Manufacturing Practice (GMP) procedures. Notably, $CD34^+$DCs were transfected preferentially. Increasing mRNA concentration had a minimal effect on the percentage of $EGFP^+$ $CD34^+$DCs. Similar results were found in our human blood DC experiments (Turtle et al) and mouse bone marrow DCs [11]. However, increasing mRNA concentration increased the amount of translated protein, which may necessitate further titration to optimize individual high-avidity TAA-specific CTL generation. We anticipate finding that $CD34^+$DCs, which express transfected mRNA at peak levels, are more desirable for CTL induction. In our 24-hour time course, EGFP expression occurred rapidly (within 3 hours of transfection), peaking after 12 hours regardless of the day on which the $CD34^+$DCs were transfected. This ongoing expression augurs well for $CD34^+$DC functionality after injection and migration in the patient. A time course performed by Van Meirvenne et al [11] using bone marrow–derived DCs showed no peak expression over 36 hours, whereas Ueno et al [23] reported that EGFP expression in adult $CD34^+$DCs was still readily detectable 24 hours after electroporation, but declined gradually thereafter. The method used to generate $CD34^+$DCs from CB is equally efficient when generating $CD34^+$DCs from bone marrow and mobilized peripheral blood (data not shown), but further work is required to establish whether these differences in RNA

expression reflect physiological differences between different types of DC preparations.

Electroporation in the presence of RNA led to appropriate induction of costimulatory molecules, possibly due to RNA-induced toll-like receptor (TLR) activation of $CD34^+$DCs [30]. The ability of transfected DCs to present translated mRNA in the context of MHC for recognition by T cells is crucial for CTL induction. After electroporation with IVT-FMP mRNA, $CD34^+$DCs translate and present peptides, as evidenced by the specific recognition and lysis of FMP-expressing $CD34^+$DCs by the FMP T-cell clones. Most importantly, we generated antileukemic CTLs using total RNA derived from both Nalm-6 and a patient ALL xenograft [17]. The resultant CTLs were able to lyse the leukemia targets specifically. This demonstrates the potential efficacy and safety of total leukemic cell RNA in stimulating antileukemic responses.

Only 1 antigenic restimulation was required to generate a strong antileukemic response for the ALL cell line and the patient's ALL xenograft. This success compared with other approaches may relate to the heterogenous nature of our $CD34^+$DC preparation or to the use of improved electroporation conditions that allow more efficient gene delivery and hence more efficient translation and presentation of peptides. Furthermore, the $CD34^+$DCs are highly activated with increased expression of costimulatory markers only after electroporation with RNA, which means that the $CD34^+$DCs are fully activated when they encounter the T cells. Using total RNA as the antigen source has advantages as well, including a potentially greater capacity to activate TLRs [30], and thereby DC-mediated CTL induction, and also to present a wide variety of known and unknown TAAs, which may increase target cell lysis.

Poor immune reconstitution and the potentially limited period before early leukemic relapse after CB HSCT makes active donor DC vaccination to generate an in vivo anti-ALL CTL response a difficult prospect. We propose that $CD34^+$DCs may be used to generate anti-ALL specific CTLs in vitro for subsequent passive immunotherapy posttransplantation. However, adoptive immunotherapy using total RNA as the source of leukemic antigen may be a double-edged sword, with the potential for generating CTLs against known and unknown TAAs but also the potential to generate alloreactive CTLs and induce GVHD. Survivin is an antiapoptotic protein expressed in leukemia and other malignancies [19], and the use of specific survivin mRNA or other leukemia TAAs may become a more defined posttransplantation immunotherapy strategy. For this reason, we included survivin as a target and showed that anti-ALL xenograft CTLs were able to lyse survivin-loaded $CD34^+$DCs. These observations suggest that survivin is a valid target antigen for ALL and that the DC culture and electroporation protocol generates a polyclonal antileukemic CTL population that preferentially recognizes leukemic cells. These results suggest that our $CD34^+$DC-mediated antileukemic CTLs are more likely to induce a GVL effect as opposed to a GVHD effect.

In summary, we have defined conditions for the very effective RNA transfection of $CD34^+$DCs to generate antileukemic CTL activity. Current work is focusing on testing the in vivo efficacy of $CD34^+$DC-mediated antileukemic CTLs in a NOD-SCID mouse model of primary human leukemia. These data will pave the way for the first phase I clinical trials of post-CB transplantation immunotherapy to treat leukemia relapse [31,32,33,34,35].

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